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Demonstration of Multiple HPV Types in Laryngeal Premalignant Lesions Using Polymerase Chain Reaction and Immunohistochemistry

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Recent evidence has shown that human papillomavirus (HPV) is involved in both the development of carcinoma and in premalignant mucosal lesions of the oral cavity. This study examined the relationship of HPV infection to some pathological features in precancerous lesions of the larynx, not examined extensively so far. Fifty formalin-fixed paraffin-embedded tissue sections containing human laryngeal precancerous lesions were screened for the presence of HPV infection by polymerase chain reaction, and for capsid protein expression by immunohistochemistry with polyclonal antibody directed against the L1 protein. The presence of HPV DNA was detected in 28 of 50 specimens (56%), including 9/12 cases with mild dysplasia (75%), 3/6 cases with moderate dysplasia (50%), and 7/11 cases with severe dysplasia (64%). Multiple HPV infections, containing two or three types, were detected in 17 of the 28 HPV-positive lesions (60%). Of 21 cases with keratosis and no dysplasia, 11 were positive for HPV DNA (52%) and 4 showed L1 staining (36%). By contrast, L1 positivity was revealed only in two lesions with moderate dysplasia, confirming that fully productive HPV infection is strictly dependent on epithelial differentiation and surface keratinization. The probability that HPV is a cofactor in the malignant progression of these lesions is suggested by the fact that 3/4 patients who developed cancer within 50 months were positive for HPV DNA. ***J. Med. Virol. 59:110–116, 1999.***

KEY WORDS: HPV; PCR; dysplasia; keratosis; larynx; L1

INTRODUCTION

Human papillomaviruses (HPVs) are small circular DNA viruses of the *Papovaviridae* family that have been implicated in the genesis of benign and malignant epithelial tumors in a variety of body sites covered by mucosa or skin, such as the cervix and anogenital region, as well as in carcinoma of the upper respiratory and digestive tracts [Popper et al., 1994; Shen et al., 1996; Zur Hausen and DeVillia, 1994; Zur Hausen, 1996].

In particular, among the 77 different HPV types so far identified, some, notably HPV-6 and HPV-11 are associated with benign lesions; HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, and possibly other types have been linked to the pathogenesis of anogenital cancer and malignant lesions of the upper respiratory and digestive tracts [Mounts et al., 1982; Terry et al., 1987; Clayman et al., 1994; De Villiers, 1994]. More than 90% of cervical cancers contain these "high-risk" HPVs [Karlsen et al., 1995; Singer et al., 1995]. Viral DNA integration in these cancers is thought to result in increased expression of E6 and E7, the two major viral transforming genes that have been shown to interact with and inactivate the tumor-suppressor gene products p53 and pRb, respectively, and to immortalize human epithelial and fibroblastic cells as well as rodent fibroblasts [Lehr et al., 1988; Desmond et al., 1994; Kubbutat and Vousden, 1996; Lòpez-Amado et al., 1996].

Recurrent respiratory papillomatosis (RRP) is the most common benign tumor of the larynx associated with HPVs and affects both children and adults [Jones et al., 1984]. The polymerase chain reaction (PCR) has detected HPV DNA in >90% of these lesions, with tissue material of sufficient quality [Lie et al., 1996]. Premalignant laryngeal lesions are defined as morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart, and

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are known clinically as leukoplakias or erythroplakias [Jones et al., 1984]. Their malignant potentials vary considerably and may coexist in a single lesion so that pathological examination is indispensable for a clear diagnosis and predictive judgment. Upper oral premalignant lesions are generally regarded as due to several factors, especially tobacco and alcohol, and synergistic or cooperative interaction of HPV infection with these factors may play a role in the progression to cancer [Yeudall and Campo, 1991; Marshall et al., 1992; Balaram et al., 1995; Cruz et al., 1996]. Detection of HPV DNA in several of these precancerous lesions is unequivocal evidence that HPV infection is involved. For the oral cavity, there is a strong correlation between HPV 16, 18, highly dysplastic lesions, and invasive squamous cell carcinoma [Multhaupt et al., 1994; Donofrio et al., 1995; Fidalgo et al., 1995]. Because there are no clear criteria for distinguishing cases in which HPV is associated with laryngeal lesions with low, moderate, or severe dysplasia, it was decided to investigate such lesions by PCR to understand better the role of viral infection in the development of premalignant laryngeal lesions and to confirm productive infection by immunohistochemistry.

In this study, primers amplifying a conserved portion of the E1 gene were used for PCR detection of HPV genotypes on archival DNA extracted from 50 paraffin-embedded precancerous laryngeal specimens. Immunohistochemical staining of tissue sections with an antibody that recognizes a group-specific capsid antigen common to most of the papillomaviruses reported was used to demonstrate virus replication. Correlations were also sought between the presence of HPV genus-specific antigen and HPV type-specific DNA and the clinical and histological manifestations, and between laryngeal HPV infection and later malignant transformation and its well-known co-factors.

MATERIALS AND METHODS

Biopsy Specimens

Samples were selected from lesions diagnosed as dysplasia or keratosis of the laryngeal epithelium from patients attending the Otolaryngology Clinic, Ospedale Maggiore Novara, Italy, between 1993 and 1997. Dysplasia was graded as mild, moderate, and severe.

Of the 50 samples containing precancerous lesions, 41 were from males and 9 from females aged 28–86 years (mean, 60 years): 21 keratosis, 29 dysplasias (12 mild, 6 moderate, 11 severe). All samples were from paraffin-embedded archival material fixed in 10% neutral formalin at microlaryngoscopy. Six or seven 10-μm thick sections from the paraffin blocks were cut and processed as described below. Particular care was taken to avoid sample to sample contamination by changing gloves and cleaning microtome blades and tweezers with xylene.

DNA Extraction

A modified version of the method of Wright and Manos [1990] was used for DNA extraction. Paraffin was removed by two extractions in 1 ml of xylene for 30 min at room temperature and samples were centrifuged at 13,000 rpm. The supernatant was removed. The pellet was washed twice with ethanol to completely remove the solvent, dried at 55°C, dissolved in 300 μl of digestion buffer (50 mM Tris pH 8.5; 1 mM EDTA; 0.5% Tween 20) containing 200 μg/ml of Proteinase K (Boehringer) and incubated for 24 hr at 55°C. Proteinase K was then heat inactivated by incubating the samples for 8 min at 95°C. Samples were clarified by centrifugation and the supernatant was used for the amplification.

PCR

Sample DNA (20 μl) from each extraction was used for PCR on a DNA thermal cycler (Touch Down,

HPV type		Sequence (5'-3')	Genomic location	Size of amplified product (base pair)
6	A	GCTAATTCGGTGCTACCTGT	401-420	140
	B	CTGGACAACATGCATGGAAG	521-540	
11	A	CGCAGAGATATATGCATATG	221-240	90
	B	AGTTCTAAGCAACAGGCACA	291-301	
16	A	TCAAAAGCCACTGTGTCCTG	421-440	120
	B	CGTGTTCTTGATGATCTGCA	521-540	
18	A	ACCTTAATGAAAAACCACGA	371-390	100
	B	CGTCGTTGGAGTCGTTCCTG	451-470	

Fig. 1. Specific primers complementary to E6 region of human papillomavirus (HPV) type 6, 11, 16, and 18.

TABLE I. Clinical, Pathological, Molecular Biology, and Immunohistochemical Data of 50 Patients With Laryngeal Precancerous Lesions

Case no.	Year of biopsy	Keratosis	Dysplasia	HPV infection	L_1 staining	Relapses prior	Relapses post
1	1996	+	–	–	–	2	1
2	1995	+	–	+	+	1	2
3	1996	+	–	+	+	1	/
4	1996	+	–	+	–	1	/
5	1996	+	–	–	–	/	/
6	1995	–	Mild	+	–	1	/
7	1993	+	–	+	–	/	/
8	1993	+	Mild	–	–	/	1
9	1993	+	–	–	–	/	/
10	1994	+	–	+	–	2	/
11	1994	–	Moderate	+	++	1	/
12	1996	+	–	+	–	1	/
13	1996	+	–	–	–	1	/
14	1996	+	–	–	–	/	/
15	1996	+	–	+	++	/	/
16	1996	+	–	–	–	/	/
17	1996	+	–	–	–	/	/
18	1997	+	–	–	–	/	1
19	1997	+	–	–	–	/	/
20	1997	+	–	–	–	/	/
21	1997	+	–	und	und	/	/
22	1997	+	–	+	+++	/	1
23	1996	+	–	+	–	2	1
24	1997	+	–	–	–	6	/
25	1995	–	Mild	+	–	1	/
26	1996	+	Mild	+	–	/	/
27	1995	+	Mild	+	–	1	/
28	1994	+	Mild	+	–	/	/
29	1994	+	Moderate	+	+	2	5
30	1993	+	Mild	+	–	/	/
31	1994	+	Severe	+	–	/	1
32	1996	+	Mild	–	–	1	/
33	1996	+	Mild	–	–	/	/
34	1996	+	Mild	+	–	/	/
35	1997	+	Mild	+	–	/	/
36	1997	+	Mild	+	n.d.	/	/
37	1995	–	Moderate	–	–	/	/
38	1996	–	Moderate	+	–	/	1
39	1994	+	Moderate	–	–	/	/
40	1993	+	Moderate	–	–	/	/
41	1995	–	Severe	–	–	/	/
42	1995	–	Severe	+	–	/	1
43	1996	–	Severe	–	–	/	/
44	1996	+	Severe	+	–	/	/
45	1993	–	Severe	+	–	/	1
46	1996	+	Severe	+	–	/	/
47	1996	–	Severe	+	–	/	/
48	1997	+	Severe	–	–	/	/
49	1996	–	Severe	+	–	/	/
50	1997	–	Severe	–	–	1	/

HPV, human papillomavirus; +, ++, +++, L1 staining intensity; n.d., not done due to small amount of material left after conventional histology; und, undetected. Prior and post are in relation to the year of biopsy.

HYBAID, Teddington Middlesex, UK). The suitability of the DNA for amplification in each specimen was confirmed by successful amplification of a fragment of the β-globin gene using the primers described previously, which gave an amplified product of 260 bp [Saiki et al., 1985].

Two oligonucleotide primers, homologous to the highly conserved E1 region of the HPV genome, were synthesized in an oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). The sequence was as follows: GP1 (sense), 5′ TGGTACAATGGGCATATGAT 3′ and GP2 (anti-sense), 5′ ATAATGGCTTTTGGAATTTACA 3′ [Chen et al., 1994]. The HPV-positive specimens were subjected to another cycle of amplifications with four pairs of specific primers for the E6 region of HPV 6, 11, 16, and 18 respectively (Fig. 1) [Griffin et al., 1990]. Amplification reactions were set up in a final volume of 100 μl containing: 20 μl of the extracted sample, 1× Taq buffer (20 mM Tris-HC1 [pH 8.4]; 50 mM KCl, 2.5 Mm $MgCl_2$), 200 μM of each of the four dNTPs, 100 pm of each of the general primers; 2 U Taq DNA Polymerase (GIBCO, BRL, Bethesda, MD). The same protocol was employed for the specific primers, except that 40 pm of each primers were used. After an

TABLE II. Age, Sex, Histological Examination (Mild, Moderate, or Severe Dysplasia) and HPV Type of 28 HPV-DNA-Positive Laryngeal Biopsy Specimens

Case no.	Age/sex	Histology	GP	HPV 6	HPV 11	HPV 16	HPV 18
2	48/M	Keratosis	+	–	+	–	+
3	70/M	Keratosis	+	–	+	+	–
4	48/M	Keratosis	+	–	+	+	–
6	67/M	Mild	+	–	+	+	–
7	55/M	Keratosis	+	–	+	+	+
10	66/M	Keratosis	+	–	+	+	+
11	62/F	Moderate	+	–	+	+	+
12	75/M	Keratosis	+	+	+	–	–
15	43/M	Keratosis	+	–	+	–	–
22	68/M	Keratosis	+	–	+	+	–
23	68/M	Keratosis	+	n.d.	n.d.	n.d.	n.d.
25	67/M	Mild	+	–	+	–	–
26	71/M	Mild	+	–	+	–	–
27	35/M	Mild	+	–	+	–	+
28	63/M	Mild	+	–	+	+	–
29	72/M	Moderate	+	–	+	+	+
30	62/M	Mild	+	–	–	+	+
31	81/M	Severe	+	+	+	+	+
34	68/M	Mild	+	–	+	–	–
35	53/M	Mild	+	–	+	–	–
36	70/M	Mild	+	+	–	–	–
38	42/F	Moderate	+	–	+	+	–
42	67/M	Severe	+	–	+	–	–
44	62/M	Severe	+	–	+	+	–
45	86/F	Severe	+	–	+	+	+
46	71/F	Severe	+	–	+	–	–
47	71/M	Severe	+	+	+	–	–
49	55/M	Severe	+	+	–	–	–

HPV, human papillomavirus; n.d., not done due to small amount of material left after conventional histology.

initial "hot start" of 10 min at 85°C, DNA amplification was performed for 40 cycles, using the following thermocycle-step parameters: 94°C for 1 min to denature the DNA, 2 min at 40°C for annealing, and 90 sec at 72°C with a final extension of 10 min at 72°C.

The negative controls were samples with water replacing target DNA in the reaction mixture. The β-globin and the specific primers were run under the same conditions: initial 10 min at 85°C, each cycle 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. Then, 20 μl of the reaction mixture were removed for electrophoresis on 1% agarose (for β-globin and E1 amplification) or 10% polyacrylamide gel (for E6 amplification). The reaction products were visualized by ethidium bromide staining and photographed.

Immunohistochemistry

Five-micrometer sections floated onto glass slides coated with 3% aminopropyltriethoxysilane (Sigma, St. Louis, MO) were dried overnight at 37°C. They were dewaxed in xylene twice, 30 min each at 37°C, dehydrated twice in absolute ethanol for 15 min, and rehydrated through decreasing concentrations of ethanol. After blocking the endogenous peroxidase activity with 3% H_2O_2 in phosphate-buffered saline (PBS) for 5 min at room temperature, the tissues were permeabilized in 0.2% Triton X100 in PBS at 4°C, and incubated for 1 hr in a humid chamber with rabbit polyclonal antibody (Polyclonal anti-Bovine Papillomavirus-BPV-1, Dako), which recognizes the L1 capsid protein highly conserved among the different HPVs, diluted 1: 200 in PBS plus 5% donor serum. Afterwards, the sections were washed with PBS and incubated for 30 min with a biotinylated anti-rabbit antibody diluted 1:100 in PBS, and then with the streptavidin solution complexed with biotinylated horseradish. The colorimetric reaction was developed with 1-min incubation in a humid dark chamber in 3-amino-9-ethyl carbazol (AEC) substrate working solution until the red desired precipitate was generated. The sections were mounted with 90% glycerol in PBS after 7 sec counterstaining with Mayer's hematoxylin solution (Sigma). Sections from anal condyloma with known HPV productive infection were used as the positive controls.

RESULTS and DISCUSSION

Fifty patients aged 28–86 years (mean 60 years) with laryngeal precancerosis were included in this study (41 men and 9 women): 21 were keratosis, and 29 dysplasias (12 mild, 6 moderate, and 11 severe). Amplification of the β-globin gene was included as internal control in all cases. One sample (indicated as number 21) was excluded because DNA could not be detected in the extract. Contamination was monitored by the inclusion of appropriate negative control in each experiment. We used a PCR method with general consensus primers GP1 and GP2 to amplify the highly conserved E1 region of mucosotropic HPV types [Chen et al., 1994]. HPV positivity was not detected in clinically normal

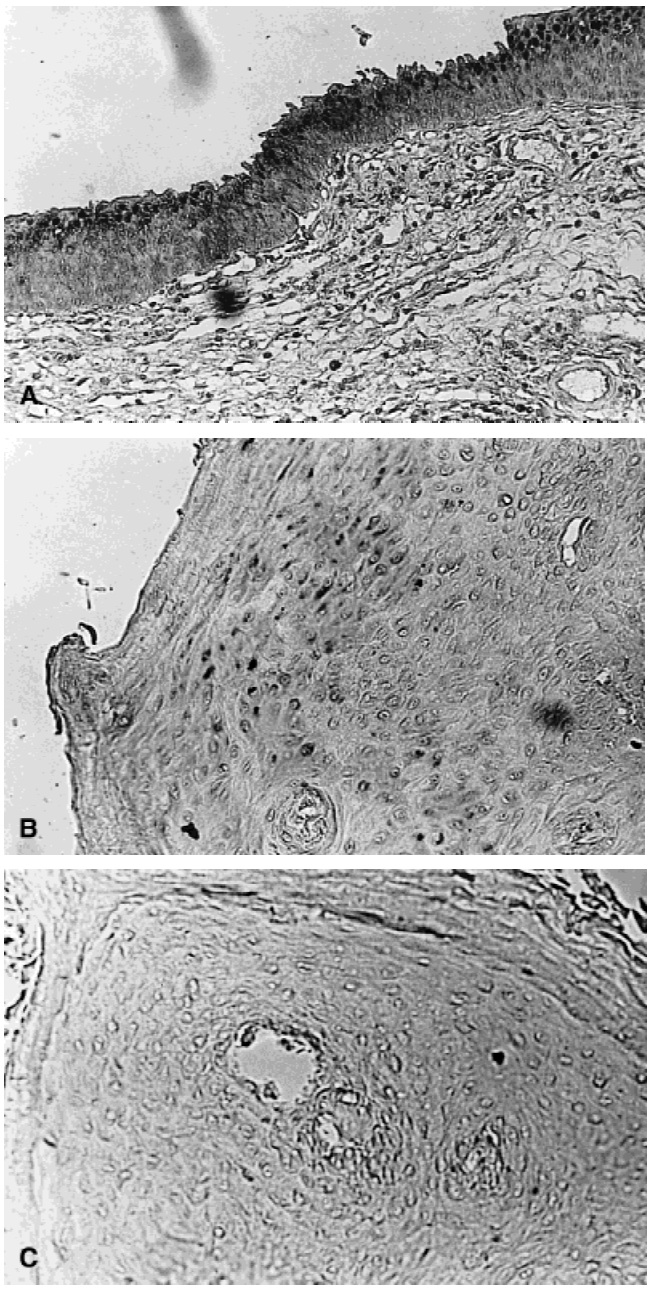

Fig. 2. Immunohistochemical staining for L1 expression. Laryngeal sections were stained using polyclonal anti-L1 antibody, followed by incubation with biotinylated secondary antibody and detection of specific antibody binding by streptavidin-peroxidase complex and 3-amino-9-ethyl carbazol (AEC). **(A)** Section from laryngeal keratosis (case no. 22) illustrating L1-positive nuclei stained darkly in the superficial layer of the epithelium. **(B)** Section from laryngeal keratosis (case no. 15) showing L1-positive nuclei distributed sparsely. **(C)** Section from a human papillomavirus (HPV)-DNA negative laryngeal hyperplastic lesion.

laryngeal mucosa of the control group (data not shown). The ethidium bromide-stained electrophoretic gel clearly indicated a DNA band at the 444-bp position in 28 cases (56%) (Table I). Further typing by type-specific PCR revealed that HPV 11 was present in 8 of 21 keratosis (38%), 7 of 12 mild dysplasias (58%), 3 of 6 moderate (50%), and 6 of 11 severe dysplasias (54%) (Table II). Multiple HPV infections, containing two or three types, were detected in 17 of the 28 HPV-positive lesions (60%). HPV 11 was the predominant type, in general agreement with other studies showing a similar frequency in laryngeal papillomas [Dickens et al., 1991]. HPV 16 was detected in 5 of 21 keratosis (23%), 3 of 12 mild dysplasias (25%), 3 of 6 moderate (50%), and 3 of 11 severe dysplasias (27%) (Table II). HPV 18 was detected in 3 of 21 keratosis (14%), 2 of 12 mild dysplasias (16%), 2 of 6 moderate (33%), and 2 of 11 severe dysplasias (18%). HPV 6 was detected in 1 of 21 keratosis (5%), 1 of 12 mild dysplasias (9%), 0 of 6 moderate, and 3 of 11 severe dysplasias (27%), and was the least frequent HPV type (Table II). This percentage (56%) of HPV positivity is higher than in laryngeal premalignant lesions reported previously [Fouret et al., 1995]. This discrepancy probably depends on the type of tissues examined and the detection methods.

The age, sex, and cigarette consumption data showed no significant differences in the frequency of HPV infection; five positive cases (nos. 7, 12, 25, 29, 46), in fact, were from nonsmokers free from other oncogenic risk factors, suggesting that HPV may play a crucial role in the genesis of laryngeal hyperplastic lesions.

All the HPV-positive samples were then tested for the presence of the L1 capsid antigen. The distribution of positive nuclei varied from one specimen to another. A uniform pattern of numerous antigen-positive cells in the upper layer of the epithelium was usually observed (Fig. 2A). In other instances, antigen-positive nuclei were distributed sparsely and a few positive cells were observed in patchy areas of the upper epithelium (Fig. 2B). However, the reaction was highly specific, and interpretation was not difficult. Of the 28 samples, 6 were L1 positive: 4 keratosis, 0 mild, 2 moderate, and 0 severe dysplasias (Table I). L1 staining is well visible in some of the superficial, highly differentiated keratinized cells of the lesions where the replicative cycle is complete, and it is mainly intranuclear (Fig. 2A). The complete replicative cycle, in fact, takes place only in highly differentiated squamous epithelium. Even in lesions, these viruses replicate actively only in the keratinized cells. This explains the high frequency of L1 staining in keratosis (36%), compared with the less differentiated dysplasias. Furthermore, L1 positivity was stronger and detectable in more cells in keratotic lesions. Three of four L1-positive keratoses relapsed. Immunohistochemical analysis of the subsequent recurrences revealed that L1 positivity was maintained and hence the productive infection (data not shown). This significant number of relapses is therefore related to the HPV productive infection, which does not allow complete surgical eradication of the infectious agent.

The low frequency of papillomavirus capsid antigen L1 in more severe dysplasias was not unexpected. This finding does not detract from the possibility that HPV may play a role in the malignant progression of these lesions. In fact, the capsid antigen would most likely be

TABLE III. HPV Detection in Patients Developing Cancer

Case no.	Epithelial dysplasia	HPV type	L1 staining	Time of cancer development (months)	Smoking (cig./day)
38	Moderate	11, 16	—	20	10–20
41	Severe	—	—	12	>20
42	Severe	11	—	30	>20
45	Severe	11, 16, 18	—	50	10–20

HPV, human papillomavirus.

present if productive infection of the cells occurred [Laimins, 1996]. By contrast, when the viral genome is present and integrated in the DNA of the transformed cells, it does not express viral structural antigens. The viral DNA is present in the less differentiated cells below, but here it is unable to express itself because there is no production of viral capsid antigens. Of the 23 recurrences observed after surgical excisional biopsy, 16 were HPV-DNA positive: type 11 in 15 samples, type 16 in 10, and type 18 in 7, whereas type 6 was detected in only 2 cases. Of these, 5 contained 2 or 3 HPV types, including both types 16 and 18, suggesting that multiple HPV infection can occur in the same lesion (Table II) [Lin et al., 1997].

The clinical and histological characteristics of the patients who developed cancer are listed in Table III. Four patients, one with moderate and three with severe dysplasias, developed squamous laryngeal cancer within 12–50 months from the onset of the premalignant lesion. Three cases were HPV positive (75%); oncogenic HPV types 16 and/or 18 were present in two samples, and HPV 11 in three. In case 42 only HPV 11 was detected. According to the literature, this result is further evidence of the direct involvement of HPV infection in the malignant progression of these lesions.

The study reveals that HPV infection is relatively frequent (56%) in precancerous lesions of the larynx. In contrast, expression of L1 protein is found only in a few lesions with a more differentiated epithelium. Although the sample size may be too small for definitive conclusions, from the results some speculations may be drawn. HPV-positive premalignant lesions of the larynx, as shown for oral lesions [Nielsen et al., 1996], harbor a relatively high percentage (57%) of HPV 16–18 type, known to be associated with malignant transformation. Positivity to L1 staining is correlated with keratosis recurrency, reinforcing the hypothesis that virus replication and virion production is not associated with cancer development.

It has been shown that p53 protein may accumulate at an early stage in head and neck carcinogenesis [Fouret et al., 1995; Yeudall et al., 1995]. A better understanding of the role of HPV infection in carcinogenesis will be obtained when the status of other genes, such as p53 and pRb, whose products cooperate to regulate cell proliferation, is assessed [von Knebel-Doeberitz et al., 1988; Bartek et al., 1997].

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